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## **Function of Male Sexual Signaling in the Brief Squid (*Lolliguncula brevis*)**

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Function of Male Sexual Signaling in the Brief Squid (*Lolliguncula brevis*)

by

Lauren A. Piperno

Submitted in partial fulfillment  
of the requirements for the degree of  
Master of Arts Animal Behavior & Conservation Hunter College  
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Thesis Sponsor:

12/21/15

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### Abstract

Sexual selection results when competition and mate choice exist among members of one sex for access to the opposite sex in order to maximize mating success. Typically males possess more elaborate weapons and ornaments as a result of male to male competition and female mate choice; however, elaborate female ornamentation may evolve under certain circumstances. In these cases, the role of the less elaborate male sexual signaling is not well understood. This study explores the role of male signaling in reproduction in the brief squid, a species with female biased dimorphism and more elaborate female signaling. I compared male signaling in the presence of a female, a male, both sexes, or alone and found that male brief squid produce specific signals primarily in the presence of a female; however, the time males spent signaling did not significantly correlate with the number of sexual contact events. Tests of females' responses to males signaling showed that females spend more time close to males during male signal production, but did not change their orientation to enable mating. Females reduced their own signaling during the production of male signals indicating an awareness of the male signals. It is tempting to assume that the purpose of male sexual signaling is to attract mates, but ornamentation may exist in both females and males that serve purposes other than to attract a mate. Male sexual signaling is clearly directed at females, yet the precise function remains elusive.

*Keywords:* mate-choice, ornamentation, sexual selection, signaling

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Function of Male Sexual Signaling in the Brief Squid (*Lolliguncula brevis*)

Primary sexual characteristics are those structures and organs necessary for reproduction, while secondary sexual characteristics, typically referred to as ornaments, are phenotypic traits or behaviors that distinguish the sexes within a species, but are not part of the reproductive system. Ornamentation includes such characteristics as body size, plumage, coloration, signals and various displays, which generally give an individual an advantage in combat or courtship (Darwin, 1871). The evolution of such exaggerated ornamentation in males is typically ascribed to female mate choice and weapon evolution to male-to-male competition; however, when elaborate ornamentation evolves in females, its function is not always obvious (Clutton-Brock, 2007). There is evidence that selection for secondary sexual characteristics may operate differently in both males and females under specialized conditions (Bonduriansky, 2000; Clutton-Brock, 2007; Tobias et al., 2012). This study aims to examine the function of male signaling in brief squid, a species with female-biased sexual size dimorphism and elaborate female signaling.

Darwin found that males generally possessed more elaborate weapons, coloration and ornamentation than females and that these secondary sexual characteristics evolve to enhance reproductive success rather than to improve survival. In Darwin's sentinel work, *The Origin of the Species* (1859), he described sexual selection as an essential component of natural selection stating:

When males and females of any animal have the same general habit of life, but differ in structure, color, or ornament, such differences have been mainly caused by sexual selection: that is, by individual males having had, in successive generations, some slight advantage over other males, in their weapons, means of defence, or charms, which they

have transmitted to their male offspring alone (p. 133).

In *The Descent of Man*, Darwin (1871) further refined sexual selection as a process, “that depends on the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction” (p. 88).

The classical theory of sexual selection operates on the basis that sperm are less costly to produce than eggs and that males but not females increase their fitness by mating with multiple individuals (Bateman, 1948). Since males generally invest less in gamete production and parental care, there are generally many more sexually active males ready to mate than there are receptive females at any given time (Trivers, 1972). This asymmetry causes a male-biased operational sex ratio to develop, which results in intrasexual competition among males for female mates and allows females to be more choosy during mate selection (Clutton-Brock, 2007; Rubenstein, 2009).

Ornamentation may indicate the quality of the mate. One way females choose mates is by evaluating male quality based on certain secondary sexual characteristics that may convey information about the males' individual fitness, genetic quality, immunocompetence, or parasite burden (Møller & Petrie, 2002). One highly studied example of male ornamentation and female mate choice involves the peafowl, *Pavo cristatus*. Several investigators have studied the males' elaborate train by looking at the number of eyespots, train symmetry, and train length and found a relationship between train features and mating success (Petrie et al., 1991; Yasmin & Yahya, 1996). Møller & Petrie (2002) wanted to identify precisely what information train features convey about male fitness. In a study of immune function in a group of male peacocks, they found train length, and not size or number of ocelli, correlated positively with one of the three aspects of immune function studied: cell mediated immunity. They concluded that components

of sexual signals may provide specific information about different aspects of phenotypic quality. In contrast, Takahashi et al. (2008) found no correlation between male mating success and any of the three train traits.

Loyau et al. (2008) state that such contradictory findings are important because researchers need to look beyond the usual sources of experimental inconsistencies such as differences in methodology, small sample sizes, and experimental versus wild environments for alternative explanations. They propose that other explanations should be considered when facing contradictory findings such as: divergence of behaviors among populations within a given species, atypical variations in male traits, female preferences between populations, and plasticity in female mate-choice. Peacock studies are valuable because they generate various theoretical possibilities to explain evolution of elaborate male ornamentation, highlight potentially subtle differences in information conveyed by sexually selected traits, and expose the difficulty in determining which signal, among many, may be most relevant for female mate choice (Møller & Petrie, 2002). Regarding the present study on the function of male signals, these cautions may be particularly relevant.

Size is an important characteristic of ornamentation in mate selection, and in many species, males are larger and showier than females; however, there are examples where males may be smaller because females prefer smaller males. Griffith et al. (1999) showed that female house sparrows, *Passer domesticus*, do not always choose the largest or most ornamented males. They showed that in a certain population of house sparrow, females selectively mated with, less showy males, (as determined by measurements of a badge of black feathers), and these males were more likely to be polygamous and produced a higher number of viable offspring. They postulate that, since this is not the case in other species of house sparrows, the most likely

explanation is that female preferences are plastic and adaptive and that females select those males that can provide what is of benefit to them at any given time whether it is breeding locations, nests sites, or an assist in parental duties. Szekely et al. (2004) analyzed size dimorphism in some species of shore birds, including some sandpipers and snipes, and found that the size of the males is related to specific fighting behaviors. He found that males who fight on the ground and depended upon strength are generally larger than females, and males who engage in aerial fighting depended more upon maneuverability and quickness and are typically smaller than the females. These studies show that multiple factors may influence male-biased sexual dimorphism, and not all cases of female-biased size dimorphism indicate sexual selection is acting on females.

The evolution of sexually selected male traits is generally thought to occur over many generations; however, Chaine & Lyon (2008) performed a study that showed female preferences are not static, and that flexibility of mate choice can vary from year to year. They performed a longitudinal study over five years on the lark bunting, *Calamospiza melanocorys*, and found that sexual selection of certain male traits—specifically beak size, body size, residual mass (a measure of condition dependent body size) (Moya-Laraño et al., 2008) and five separate plumage measures of body and wing feather color varied and changed over time, and even reversed, to favor traits in males that were associated with the best reproductive outcomes. They concluded that, “plasticity in choice and concomitant reversals in mating patterns across time may weaken the strength of sexual selection and could maintain genetic variation underlying multiple sexual ornaments” (Chaine & Lyon, 2008, p. 459).

### **Female Ornamentation**

Sexual selection theories explain most instances of male ornamental trait evolution as a

result of male-to-male competition and female mate-choice; however, it does not explain many instances of mutual or elaborate female ornamentation (Clutton-Brock, 2007), or female only ornaments, specifically those displayed only during breeding (Amundsen, 2000; Chan et al., 2009). The discovery of many examples of female ornamentation that do not fit neatly into the conventional sexual selection theory has led to formulation of other theoretical possibilities.

Tobias (2012) reviewed the three most widely held views to explain female ornamentation: correlated inheritance (Darwin's explanation), female sexual selection (females are subject to the same selection pressures as males), and social selection (West-Eberhard's theory that females typically compete more for ecological resources than mates). Darwin stated that ornamental traits in males evolved through male-to-male competition for females, but believed that ornaments evolved in females as a result of a shared inheritance with males, rather than by sexual selection. Although these traits are adaptive in males, they are not always adaptive for females (Tobias, 2012). The second theory is that although female sexual selection forces are operating less frequently and only under particular circumstances, females are under the same sexual forces as males and that ornamentation is sexually selected and adaptive in the same way as it is for males (Tobias, 2012). The third theory, social selection, is that females generally need to compete more strongly for environmental resources such as food, shelter, and breeding grounds than they do for mates and that these conditions vary episodically. As a result, the secondary traits that evolve in females are more fluid (such as color changes) and are more geared toward survival than breeding and should be considered more in the framework of natural selection than sexual selection (Tobias, 2012).

Modern evolutionary biologists recognize that Darwin's theory of shared or correlated inheritance does not offer an adequate explanation for most examples of female ornamentation,

and find many examples of sexual selection operating in females in much the same way as it does in males. Females may evolve more elaborate sexual characteristics than their male counterparts, such as increased body size, brighter coloration, elaborate courtships signals, and exhibit aggressive behaviors to select their mates due to the same forces of sexual selection that operate in males, especially when there is competition between females for breeding opportunities or when male mate choice exists (Clutton-Brock, 2007). In group-living species with an unequal sharing of reproduction (reproductive skew) as well as in species where females dominate, the selection on traits used in intrasexual competition for access to mates (sexual selection) or for other resources linked to reproduction should be intense for females and therefore selection should favor development of elaborate female traits (Clutton-Brock, 2007). Females show elaborate ornamentation when female rank correlates with breeding opportunities as in some social mammals such as the Kalahari meerkats (*Suricata suricatta*) (Clutton-Brock, 2007) and the spotted hyena (*Crocuta crocuta*) (Holekamp et al., 1996). In polyandrous birds, such as the African jacana (*Actophilornis africanus*), males assume parental responsibilities and females compete for males; as a result, females are often larger than males (Clutton-Brock, 2007). Amundsen et al. (2000) showed that male mate choice selects for female coloration in a small marine fish, the two-spotted gobies (*Gobiusculus flavens*). They found that the males directed far more courtship displays toward and preferred to mate with more colorful females. Szekely et al. (2006) found that among some polygynous or promiscuous species of shorebirds, mating competition contributed to size dimorphism: when the male competed for females, males were large; whereas in those species where females competed for males, there was female-biased dimorphism. Mutual mate choice may also lead to an evolution of similar ornaments in males and females. In cooperative breeding African starlings (*Sturnidae*) there is similar competition

for mates and resources in both sexes and, as a result, phenotypic differences are diminished with reduced dimorphism in plumage and body size (Rubenstein & Lovette, 2009).

One way that female ornamentation differs from male ornamentation is that it can indicate changes in sexual receptivity. Baird et al. (2004) studied reproductive coloration in female collared lizards, (*Crotophytus collaris*) and found that female coloration in that species develops to indicate receptivity and to stimulate courtship from males rather than to just signal rejection of courtship to males. In his research on lizards, Weiss (2002) showed that males were preferentially drawn toward more highly colored (orange) females. He reports that research in lizards demonstrates little evidence for female mate choice or for male signals that predict male phenotypic condition, but found that female-specific reproductive coloration is widespread. Weiss (2006) studied the striped plateau lizard, (*Sceloporous virgatus*), and found that the size and saturation of the female body color signal peak at the time of ovulation, which indicates optimal receptivity and also communicates several aspects of female quality, including better body condition, larger eggs, and fewer mites. Weiss concluded that female specific color is a condition dependent signal that serves as a phenotypic correlate of female quality and may be maintained by sexual selection.

West-Eberhard (1983) proposed a theory of social selection and noted that competition for non-sexual resources involves similar ornamentation: displays, signals, weapons, and behaviors that evolve with sexual selection, and these characteristics will “be favored” whenever they lead to increased fitness of the individual or improve access to breeding sites, food or other limited resources (Tobias et al., 2012, p. 2275).

Acknowledging that there is no consensus among evolutionary biologists to explain all instances of elaborate female ornamentation, Tobias et al. (2012) favor the general framework of

West-Eberhard's social selection theory to include all forms of social competition for non-sexual resources. They offer migratory birds as the best example to support social selection: individuals of both sexes readily resolve conflicts over food or territory without actually fighting and use their traits to convey information about their condition or fighting prowess to achieve a desired outcome. Tobias et al. (2012) believe that social selection is a particularly useful framework to explain ornamental trait development because many traits evolve that are used in competition for resources and are not directly linked to reproduction, and it allows for certain traits to function in multiple contexts in different ways. Placing sexual selection inside the framework of social selection eliminates the classification difficulty that arises when trait evolution cannot be clearly tied to reproduction (Tobias et al., 2012).

Clutton-Brock (2009) agrees that traits which increase a female's intrasexual competitive advantage to secure resources may be more important in the production of viable offspring than that achieved by traits that influence male selection of females during mating. Citing the example of the dung beetle (*Ontophagus taurus*): a species with mutually evolved horns that are used by females for digging in the dung to lay eggs and in the male for fighting, he states that it is inconsistent to attribute horn development in the male to sexual selection, while in the female, attribute horn development to social selection. He suggests abandoning the distinction between sexual and natural selection, or perhaps a less controversial and more acceptable alternative, to broaden the concept of sexual selection beyond intersexual pressures to include "all selection processes operating through intrasexual competition for breeding opportunities in either sex" (Clutton-Brock, 2009, p. 8).

Further research on species-specific ornamentation and continued debate about selection processes for secondary traits are important. One hundred fifty years after Darwin put forth his

theory on sexual selection, evolutionary biologists and ecologists continue to find species with secondary characteristics that push the boundaries of traditional theories on selection and demand further explanation for variations in ornamentation in both males and females.

### **Signaling**

One particularly interesting form of ornamentation is signaling. Signals include, but are not limited to, visual, acoustic, vibratory, chemical, electrical, and tactile displays. Signals may be part of courtship displays, convey information about the condition of the signaler, or impart information about the environment such as threats or food sources.

Sexual selection commonly causes signals to evolve greater elaboration (Akre et al., in review). Often multiple signals are employed: displays that contain signal components transmitted via multiple sensory channels are termed ‘multimodal’ signals while ‘multicomponent’ signals are confined to one modality (Scheffer et al., 1996). Møller and Pomiankowski (1993) offer three possible explanations for signals with multiple components: the multiple message hypothesis, the redundant signal hypothesis, and the unreliable signal hypothesis. The multiple message hypothesis states that each ornament offers specific information about a different aspect of the condition of the organism. The redundant hypothesis states that each ornament provides only part of the story on the health of the organism and it needs to be reinforced or elaborated upon through other ornamentations. The unreliable signal hypothesis states that some ornaments in species are no longer accurate indicators of the condition of the organism. Animals may employ multiple signals because some signals and ornaments, may be quite specific while others are redundant, obsolete, or less reliable, based on certain conditions (Møller & Pomiankowski, 1993).

Cephalopods frequently produce multi-component signals. These animals employ an

extraordinary array of visual signals as a means of communicating, and as behaviorally complex invertebrates with large brains they offer an interesting opportunity to study elaborate visual signaling. Hanlon and Messenger (1996) have written extensively on cephalopod behavior and describe how both male and female cephalopods, with the exception of the nautilus, display a myriad of patterns on their body that are the result of neuromuscular controlled dermal chromatophores. This neural control enables the animal to rapidly change its appearance into body patterns used for communication based on visual cues it receives from its environment. Chromatic components can be displayed alone or in combinations. Chromatic, postural, textural and locomotor components combine to enable all the various squid behaviors used in predation, evasion, foraging, and mating.

Numerous cephalopod investigators have developed a species specific catalog or ethogram, of reproducible and recognizable body pattern components of various chromatic, postural, and locomotor patterns and have described whether they were long lasting or fleeting (Hanlon et al., 1994; Hanlon & Messenger, 1996; Jantzen & Havenhand, 2003, Akre et al., in review). Some have also associated each pattern with specific behaviors such as whether it was used for crypsis, which is a method of camouflage, or for interspecies communication as in mate guarding or prey/predator interaction (Hanlon et al., 1994; Hanlon & Messenger, 1996; Jantzen & Havenhand, 2004). Cephalopod signals are particularly complicated to study in the wild because these animals move so quickly over a long range of water. These functions are also difficult to test experimentally as many of the signals are fleeting, but laboratory studies performed on captive individuals offer the opportunity to learn more about squid behavior and the adaptive functions of signaling.

Akre et al. (in review) found the brief squid, (*Lolliguncula brevis*), a hardy species of

squid in captivity, to be a good species for studying questions about the signaling dynamics in a species in which females have more elaborate signals than males. Akre et al. (in review) found that the female brief squid spend a greater proportion of time producing sexual signaling components than do males, and that females produced four unique sexual signaling components that were not produced by males. Female-only signal components (FOSCs) is a term coined by Akre et al. (in review) to describe the four unique chromatic components (Dark Arms Up, Lateral Blush, Lateral Patch, and Lateral Spot) produced only by the female brief squid and never by males. They did not identify any male-only signals, but found that female signals are directed at males and indicate female receptivity, but such displays did not result in either male approach or contact. The investigators concluded that the reason for the size dimorphism and more elaborate female sexual signaling in female brief squid remains unclear. In the brief squid, both females and males are promiscuous and mate with multiple partners, but males do not provide any known resources; there is no known female biased OSR; and there is no evidence that female rank is involved in mating opportunities (Akre et al., in review). Thus female elaboration of signaling relative to males is surprising and the lack of male-specific sexual signals in this species is of particular interest. This current study attempts to evaluate the role of male sexual signaling that occurs in this species. Do males, despite their small size and lack of distinct signals, produce their signals to attract females to mate, and do females show a response to these male signals?

### **Current Study**

This study is designed to determine the role of male signaling in *L. brevis* under the testing conditions used by Akre et al., (in review) in their analysis of female signaling in the brief squid. We ask several questions:

**1. Which signals are sexual signals for males?** By analyzing male signal production in various social contexts, certain signals may emerge as ones that are used primarily to communicate with a mate.

**1a. Are certain signals produced only, or primarily, in the presence of females?** If so, these signals are more likely to be used in the presence of females to attract them and enhance reproductive opportunities. In contrast, if certain signals are produced primarily in either blank or same sex trials, these signals are unlikely to be involved in reproduction.

**1b. Does the proportion of time males spend signaling correlate with increased sexual contact events?** Ultimately it is the transfer of spermatophores that indicates mating has occurred, but this process is difficult to identify; however, body contact, a required precursor to mating, is easily identifiable. Analyzing the number of body contact events in relation to signal production may identify if there is a positive correlation between time spent signaling and contact events between males and females.

**2. How do male sexual signals influence mating?** Measures of female attraction to male signals may include a) the female moving closer to the male, b) orienting her body toward the male to indicate willingness to mate, or c) changing her own sexual signaling behavior.

**2a. Do male signals result in females moving closer to males?** If so, I should find females to be in closer proximity to males after signals are produced than before or during signal production.

**2b. Do male signals result in females changing their orientation toward males?** Similarly, by measuring the proportion of time females orient their mantle toward the male may show female receptivity to mating in response to male signaling. To mate, males grab the end of the female mantle and climb along it towards the mantle opening, where they deposit

spermatophores. Tentacle orientation may indicate an unwillingness to mate since that is where her powerful beak is located, which could be threatening.

*2c. Do females produce female-only signal components (FOSCs) in response to the male signals?* If the female produces a FOSC after a male chromatic component display, it may be a response to the male signal.

### Methodology

The current study analyzes videos collected for a previous study performed by Akre et al. (in review). The study subjects were collected from the bays by Port Aransas, TX during 2007-2009. Trials were conducted with 13 males who were each videotaped under four conditions as repeated measures. The focal male spent at least 2 min alone in the tank prior to the stimulus introduction. Then he was exposed to four conditions consisting of the addition to the tank of: i) no animals, ii) a same-sex squid, iii) an opposite-sex squid, iv) first a same-sex squid, then an opposite-sex squid. In condition iv), first the same sex squid was introduced, and at least 2 min later the opposite-sex squid was introduced. Each trial was videotaped for at least 5 min starting at the point of complete stimulus introduction. The details of squid collection and care are described by Akre et al., (in review). They created an ethogram for 12 common and distinct chromatic components in the brief squid and determined the proportion of males and females displaying each of the 12 chromatic components in each of the four social contexts. Females and males produce eight chromatic components in common; females produce an additional four female-only signal components; and males produce no male-only signal components. Although the brief squid is known to produce a variety of chromatic components, this study focuses on six specific chromatic components (see *Figure 1*) since observation of trials indicated they were most commonly used in sexual interactions and for reproduction.

I analyzed the videos of 37 trials that could provide signaling information on the male squid. The trials consisted of 12 opposite sex (female) trials, 6 same sex (male) trials, 12 male and female (mixed) trials, and 7 blank trials. A total of 13 different males were used in these trials. Some males could not complete all conditions due to injury, illness or death.

In each trial, I focused on the male and recorded every instance of male production of six target chromatic components: Dark Body (DB), Dark Fin Detail (DFD), Shaded Eyes (SE), Thick Mantle V (TMV), Thin Mantle Rim (TMR), and Anterior Mantle Bars (AMB) that were displayed in the four social contexts, the time points each display occurred, the duration of each chromatic component, and the overall duration of each video. If the signal duration was very brief, less than one second, it was calculated by employing a frame-by-frame analysis. In this case, the number of frames during which the signal was displayed was divided by 15 (the total number of frames in one second). This method provides a consistent way of reporting data in instances of rapid signals.

### ***1. Which signals are sexual signals for males?***

#### ***1a: Are specific male signals produced only, or primarily, in the presence of females?***

In order to determine whether male signals are produced only, or primarily, in the presence of females, I reviewed 37 trials and calculated the proportion of time that males displayed the six chromatic components (DB, DFD, SE, TMV, TMR, AMB) in each of the four social contexts (see *Figure 2*). I recorded the duration of each signal divided by the total duration of that video trial to yield the proportion of time that the specific signal is displayed. I summed the data from each male and found the average per male to determine the proportion of time each signal is produced in each context. This calculation is an average of all 13 males (Blank N = 7 trials, Male N = 6 trials, Female N = 12 trials, Mixed N = 12 trials).

Since the chromatic components DFD and SE had significant production in the blank trials, they were considered unlikely to be used to attract females. The remaining four signals DB, TMR, TMV, and AMB are produced only in the presence of females except for one instance, and thus we conclude they are directed at females and most likely to be sexual signals. Therefore, for the remaining questions of the study, I focus on the four sexual signals that were produced only or primarily in the presence of females.

*1b. Does the proportion of time males spend signaling correlate with increased sexual contact events?* Next, we determined whether the proportion of time that male chromatic components are displayed in the presence of a female correlates with an increased likelihood of femal body contact. To determine this, I reviewed 24 female and mixed trials with 12 individual males and calculated the proportion of time that males displayed the four chromatic components in these trials. I also calculated the number of times body contact occurred between a male and female in a trial without regard to whether contact occurred before, during, or after the male signal display. Body contact included any type of contact with the female by the male such as a grab (male wraps his tentacles around the female), touch (any type of body contact made between male and female), or mate (male transfers his spermatophores). I then analyzed whether there is a significant correlation between these two variables. For the correlation, I calculated the average proportion of time the male spent displaying the four identified sexual signals. Because each individual usually completed two trials (opposite sex and mixed sex trials), I calculated averages for each individual: the average number of body contacts and the average proportion of time signaling.

**2. How do male sexual signals influence mating?** Do male signals elicit responses in the female that indicate attraction and willingness to mate? Here I analyzed three separate aspects of female response to male signals: proximity, orientation, and production of FOSCs.

### **Female Proximity**

**2a. Do male signals result in females moving closer to males?** To evaluate whether male displays attract females to move closer to the male, I analyzed the proportion of time that the female was within one female body length of the male *before*, *during*, and *after* male sexual signaling. I recorded the female's body position 10 seconds *before* the initial male signal, *during* the signal, and ten seconds *after* the signal ended. Female body position was recorded as being within one female body length of the male, greater than one female body length away from the male, or not visible. If less than 10 seconds elapsed between signals, I employed the following method. *Before* was identified as 10 seconds before the first signal, *during* was from the start of the first signal all the way through until the last overlapping signal ended, and *after* was 10 seconds after the last signal. For overlapping signals, this meant that a *during* phase included non-signaling time between two signals that occurred less than 10 seconds apart. For this analysis, all individual and overlapping signals in both the female and mixed trials are included and were employed for parts 2a-2c. If there was signal overlap, the signals were grouped together and labeled as a single signal event. Thus, we do not separate the four male sexual signal components in this section.

The proportion of time females spent within one body length of the male *before*, *during* and *after* a male signaling event was analyzed via a Generalized Estimating Equation analysis (GEE) Estimated Marginal Means (EMM) in SPSS 23. The GEE analysis takes into account the

individual completing each event so that multiple individuals can contribute different numbers of events to the dataset.

### **Female Body Orientation**

*2b. Do male signals result in females changing orientation toward males?* To evaluate whether females orient their bodies to facilitate mating in response to male signals, I calculated the proportion of time that females oriented their bodies to present either mantle (pro-mating) or tentacles (anti-mating) toward the male *before*, *during*, and *after* male sexual signaling in both the female and mixed trials. Female body orientation was recorded as mantle, tentacles, other, or not visible *before*, *during*, and *after* a signaling event, as defined in the above section. The proportion of time females spent orienting either their mantle and or tentacles was calculated for each signal event.

For this section, 12 males completed 24 trials, for a total of 403 signaling events. The proportion of time females spent orientating either their mantle or tentacles *before*, *during* and *after* a male signal was analyzed via a Generalized Estimating Equation analysis (GEE) Estimated Marginal Means (EMM) in SPSS 23. Again, the GEE allows analysis of this dataset taking individuals into account so that multiple individuals can contribute different numbers of events.

### **Female Only Signal Components**

*2c. Do females produce female only signal components (FOSCs) in response to the male signals?* FOSC production was analyzed to determine whether male signaling influenced female production of FOSCs (2c). Here, we used 12 males and 24 trials to generate a total of 331 signal events and calculated the proportion of females that produced an FOSC during the three signal events. I looked at whether FOSCs were displayed *before*, *during* and *after* male sexual

signaling to determine whether male signaling influenced female signaling. I recorded the presence or absence of any of the four FOSCs *before*, *during*, and *after* male sexual signaling in both the female and mixed contexts by a simple *yes*, *no*, or *not visible* when the female was out of the frame. Again, the GEE allowed use of all events. To analyze the influence of time point on production of female FOSCs, we used the GEE procedure Estimated Marginal Means (EMM) in SPSS 23 to create a binary logistic model of proportion of events that included FOSCs predicted by time point.

## Results

### *1. Which signals are sexual signals for males?*

#### **Male Sexual Signals**

Figure 2 shows the proportion of time that males display the six chromatic components in the four social contexts.

#### **Body Contact Events**

Pearson product-moment correlation coefficients were computed to assess the relationship between the proportion of time each of the 12 individual males displayed the four identified sexual signals and the number of contact events in 24 female and mixed trials combined. There was no significant correlation between the proportion of time males signaled and body contact events,  $r = -0.032$ ,  $n = 12$ ,  $p = 0.92$ ; Fig 3a. Pearson product-moment correlation coefficients were also computed to assess the proportion of time males displayed the four sexual signals and number of contact events in female vs. mixed trials separately. For female trials, there was no statistical relationship between variables,  $r = 0.414$ ,  $n = 12$ ,  $p = 0.181$ ; Fig 3b. For mixed trials, there was no statistical relationship between variables,  $r = -0.163$ ,  $n = 12$ ,  $p = 0.613$ ; Fig 3c.

## *2. How do male sexual signals influence mating?*

### **Female Proximity**

To analyze the influence of time point on proximity, we used the GEE procedure to create a linear model of female proximity predicted by time point. A logistic regression with proximity as the dependent variable and time point as the predictor showed that time point was a significant factor in the model ( $N = 408$  signal events, 12 males; Wald  $\chi^2 = 159.559$ ;  $df = 2$ ;  $p < 0.0001$ ; Fig. 4). A higher proportion of time spent within one body length of the male occurred *during* male signaling events.

A pairwise comparisons calculation showed that females spent significantly more time within one body length of the male *during* ( $M = 0.763$ ) compared to *after* ( $M = 0.586$ ,  $p < .0001$ ) a male signaling event. Females also spent significantly more time within one body length of the male *during* signaling compared to *before* ( $M = 0.622$ ,  $p < .0001$ ). There was no significant difference found for four male signal events between the amounts of time females spent within one body length of the male *before* ( $M = 0.622$ ) and *after* ( $M = 0.586$ ,  $p = 0.189$ ; Fig. 4).

### **Female Body Orientation**

To analyze the influence of time point on female body orientation, we used the GEE procedure to create a linear model of female body orientation predicted by time point. In the first test of female body orientation to the male, a logistic regression with mantle orientation as the dependent variable and time point as the predictor showed that time point was not a significant factor in the model ( $N = 403$  signal events, 12 males; Wald  $\chi^2 = 0.265$ ;  $df = 2$ ;  $p = 0.876$ ; Fig. 5). In the second test of female body orientation to the male, a logistic regression with tentacle orientation as the dependent variable and time point as the predictor showed that time point was

not a significant factor in the model (N = 403 signal events, 12 males; Wald  $x^2 = 3.457$ ; df = 2; p = 0.178; Fig. 5).

A pairwise comparisons calculation showed there were no significant differences in the time females spent orienting their mantle towards males *before* (M = 0.60) and *during* (M = 0.57, p = 0.669), *before* and *after* (M = 0.60, p = 0.965) or between *during* and *after* (p = 0.608) a male signal event. There were also no significant differences in the time females spent orienting their tentacles towards males *before* (M = 0.12) and *during* (M = 0.08, p = 0.064), *before* and *after* (M = 0.09, p = 0.368) or between *during* and *after* (p = 0.626) a male signal event (see *Figure 5*).

### **Female-Only Signal Components**

In the test of female FOSC production, a logistic regression using GEE with FOSC presence as the dependent variable and time point as the predictor showed that time point was not a significant factor in the model (N = 331 signal events, 12 males; Wald  $x^2 = 4.896$ ; df = 2; p = 0.086; Fig. 6).

A pairwise comparisons calculation showed that there were no significant differences in the presence of FOSCs *before* (M = 0.06) and *after* (M = 0.03, p = 0.141) or *during* (M = 0.00) and *after* (p = 0.080) a male signal event, however females produced significantly more FOSCs *before* compared to *during* a male signal event, (p = 0.027); however it exceeds a bonferroni corrected p value adjustment.

### **Discussion**

We found that male squid produce four signals only in the presence of females, and we determined these are likely to be sexual signals. However, the proportion of time males spent signaling did not correlate with sexual contact. We tested whether signaling influenced female behavior and found that females spend more time in proximity to the male during male signaling

relative to *before* or *after*. Although the female is closer to males while males are signaling, this study does not allow us to determine whether proximity is determined by the actions of the female or male: however, females could swim away from the males if their signals acted to repel mating. Females are also more likely to present their mantle rather than tentacles to the males throughout the signaling event and do not adjust their body orientation to facilitate mating as the result of male signaling. Females also spend less time producing their own sexual signals during male sexual signaling.

We found that males do have multiple chromatic displays that are produced more frequently in the presence of females than in blank conditions and never in the same sex trials. Four displays, DB, TMV, TMR, and AMB, are produced almost exclusively in the presence of females and were therefore further evaluated as reproductive signals. Chromatic components DFD and SE are produced frequently in the blank context and are therefore unlikely to be used for reproduction. Any signal found to be exclusively, or primarily, produced in the mixed context is more likely used for inter-sexual competition, but no such signals were identified. I also analyzed whether the proportion of time that males displayed signals was positively correlated with the likelihood of female contact; however, no correlation was found when looking at the four signals identified as probable sexual signals.

When analyzing the influence of male sexual signals on female behavior, I found that under these test conditions, females tended to swim close to the male throughout the male signal events; however, females were more likely to be within one body length of the male *during* production of the display than either *before* or *after* the display.

Females were more likely to orient their mantles rather than tentacles toward the male throughout all stages of male signaling events: however, females spent an equivalent proportion

of time orienting either the mantle or tentacles throughout the signal events with no differences in phases: *before*, *during* and *after*. This could mean that females were generally ready to accept mating during the signaling events because if females were not receptive to mating, then it is more likely that they would have oriented their tentacles towards males in general. Perhaps by using sexually receptive females in the study, the effect of male signaling on orientation is less evident.

Lastly, it was found that a higher proportion of females producing FOSCs occurred *before* a male signaling event. If a female produces a FOSC before the male produces a chromatic display, then the FOSC is not considered a response to the male signal; however, if a FOSC is produced after the male display, it may be a response to the male signal. Since there was no significant difference between *before* and *after*, FOSCs were not likely a response to male signals. Females do not produce FOSCs in response to the male signal production; however, the fact that females signal less while males are signaling seems to indicate an awareness of the males' display. By dividing the male signal events into three distinct phases: *before*, *during*, and *after*, it may appear that signaling did not cause females to respond with an FOSC, when in fact from personal observation, it might be skewed by the short duration of the signal in the *during* phase since most signals were less than 10 seconds.

It is tempting to assume that male sexual signaling, in general, follows a pattern of evolving elaboration to attract mates, but there are numerous examples to show that females may evolve size dimorphism and elaborate ornamentation in response to male mate-choice or that ornamentation may exist in both females and males that serves purposes other than to attract a mate. I wanted to explore the function of male signaling in the uncommonly studied situation of a species with female-biased size dimorphism and elaborate female signaling. In the case of brief

squid in captivity, males do direct their signals at females; however, signals do not clearly function to attract females to mate. Male signals do not seem to have great impact on female behavior, although signal production does correlate with female proximity and reduced female signaling. Akre's research postulated that female signals may improve visibility in turbid water or serve as a redundant signal to allow males to better track them. If male signals do not attract females to mate, what might be their function?

Both male and female brief squid directed their signals to the opposite sex, but those signals did not result in increased contact events. Is it possible that when looking at four individual apparent male signals, it might be analogous to the findings in the peacock? Is it possible as cautioned by Moller & Petrie that there is difficulty in determining which signal, among many, may be most relevant for female mate choice? As with the peacock, it is possible that an individual male squid signal might actually be composed of smaller components? Alternatively, perhaps the signals must be produced in a pattern or sequence to attract a female to mate. There were instances of overlapping signals, but these were not looked at as a separate group.

Does the fact that males share eight of the twelve signals in common with the females and that both sexes are promiscuous mean that there might be mutual mate choice in this species? Mutual mate choice usually results in less pronounced differences in secondary sexual characteristics between the species, and one would expect to see males and females have less size differences if this were so. It is interesting that both male and female squid signal primarily in the presence of the opposite sex and that signals are directed to the opposite sex, but signals did not lead to increased matings. Perhaps it is not visual signaling that induces mating in either sex.

Why are male squid smaller than females? Are they like the house sparrows described by Griffith et al. (1999)? Does smaller male size serve a purpose, such as enhanced agility and maneuverability to follow females or for predator evasion, rather than to suggest that male mate choice is operating?

This study does not answer the question whether any specific individual male signals resulted in an increased likelihood of female contact, specifically after signal production, which would have supported the hypothesis that a signal is reproductive. I collected extensive contact data for each individual signal in the hope of identifying whether individual signals resulted in body contact events *after* the signal, but some signals were produced in very small numbers or produced primarily by very few individuals from personal observation, which limited individual chromatic component analysis. Perhaps the signals that were fewest in number or produced the least amount of time by males are most important, but that cannot be detected in this study. Much is still left to explore about the role of male signaling in this species.

Studying the function of ornamentation is important because there is still much to be learned on how selection of secondary sexual characteristics may operate differently between the sexes in various species under specific conditions. Further study is required to determine a precise function for male signals. Measured behaviors of a species in captivity may not accurately represent behavior in the wild. Squid typically travel in large numbers and matings are promiscuous in both sexes. This could have a profound influence on how signals are employed under normal mating conditions. Observation of squid in more naturalistic settings and studying other species that use more elaborate female sexual signaling may provide additional insight.

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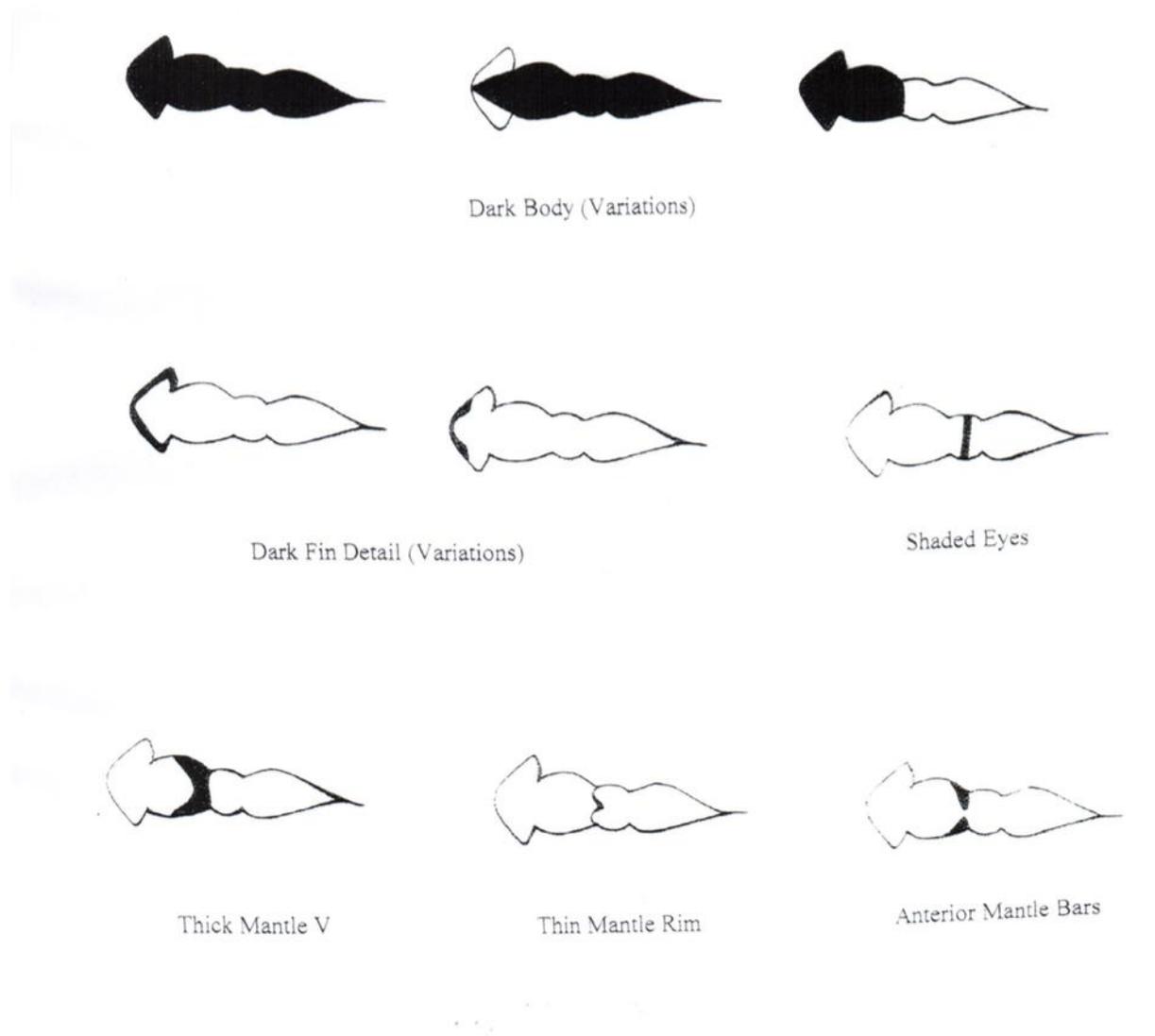
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*Figure 1.* Six chromatic components produced by male *Lolliguncula brevis*: Dark Body (DB), Shaded Eyes (SE), Dark Fin Detail (DFD), Thick Mantle V (TMV), Thin Mantle Rim (TMR), and Anterior Mantle Bars (AMB).

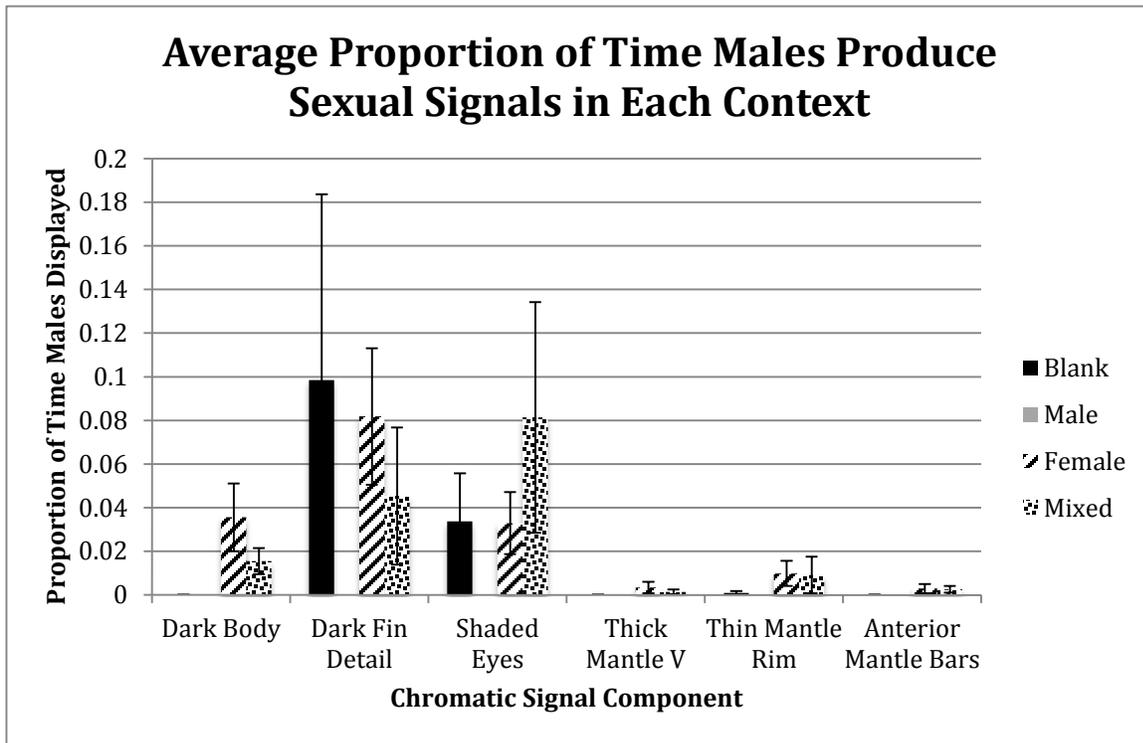
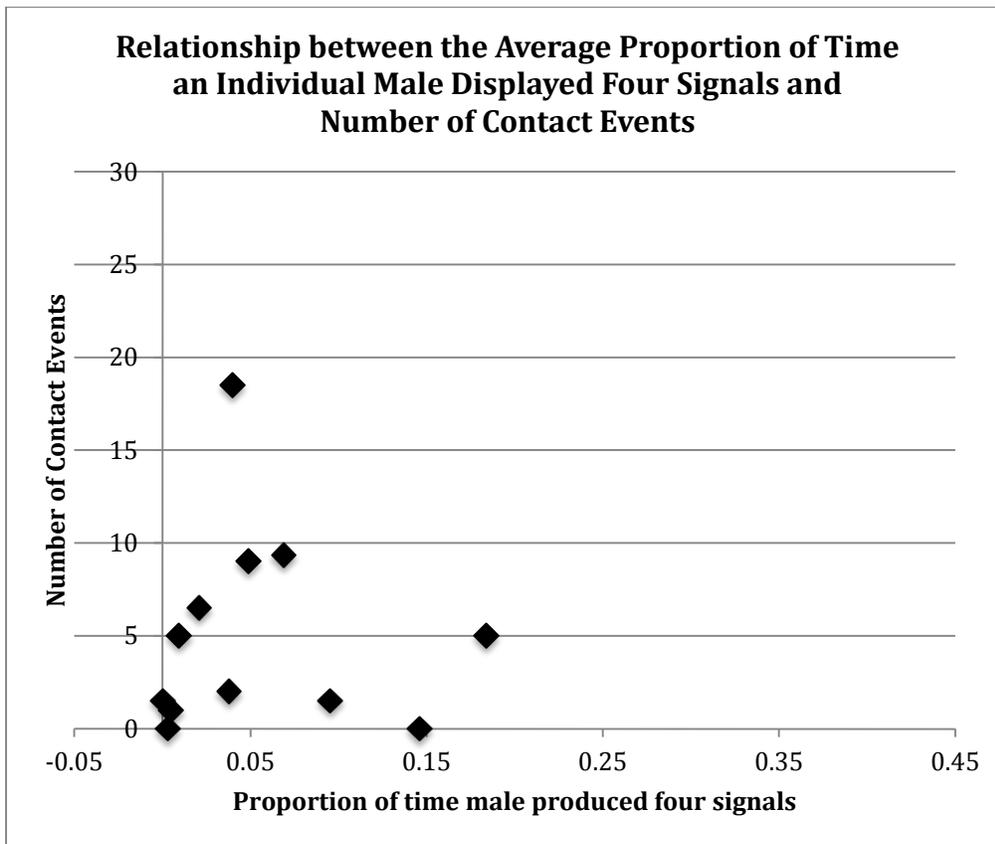
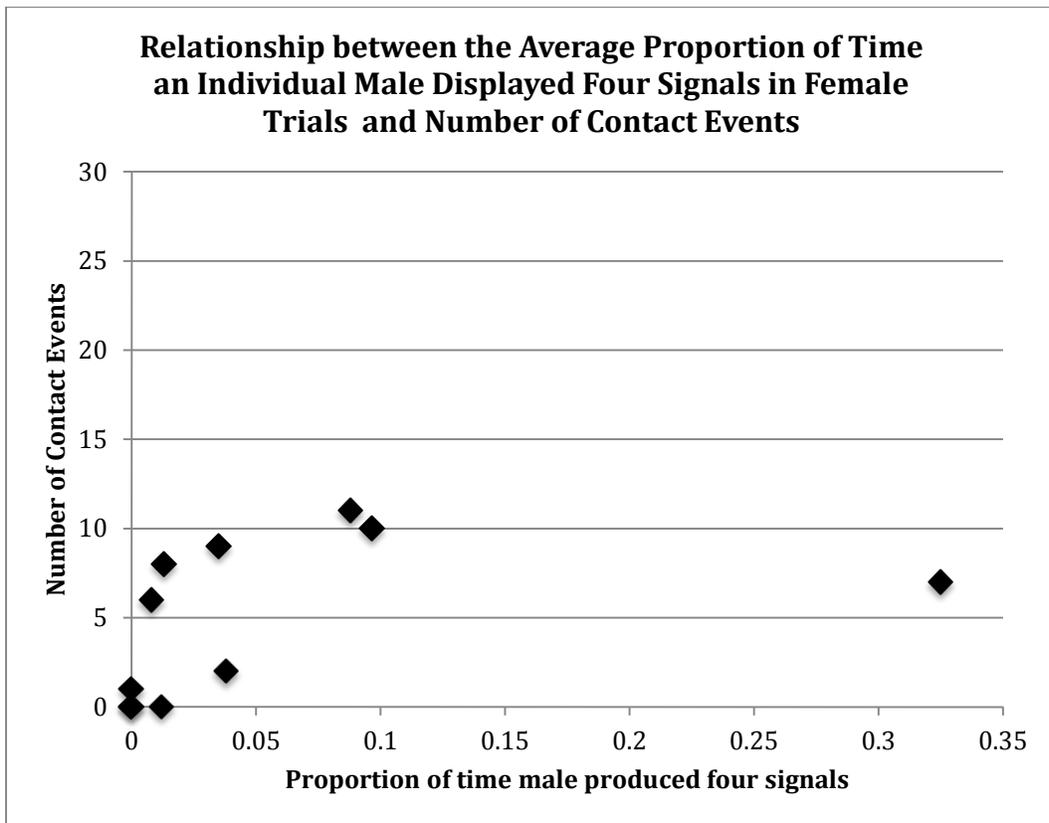


Figure 2. The proportion of time males display individual chromatic components Dark Body, Dark Fin Detail, Shaded Eyes, Thick Mantle V, Thin Mantle Rim, and Anterior Mantle Bars in four social contexts, N = 13. Standard error bars are shown around the mean.



*Figure 3a.* Relationship between the average proportion of time males displayed DB, TMV, TMR and AMB and the number of contact events. Each data point represents the average of an individual male's proportion of time spent producing four signals and average number of contact events in female and mixed contexts.



*Figure 3b.* Relationship between the average proportion of time males displayed DB, TMV, TMR and AMB and the number of contact events in female trials.

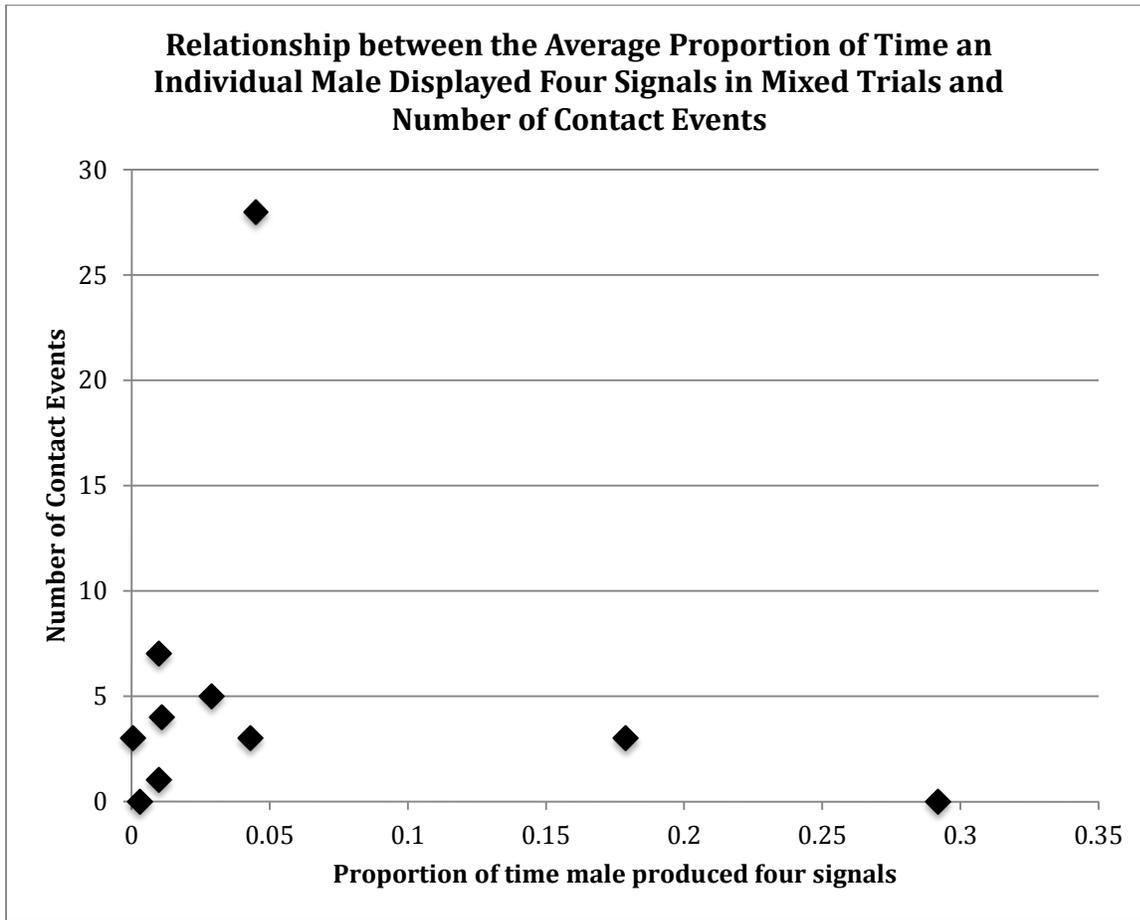
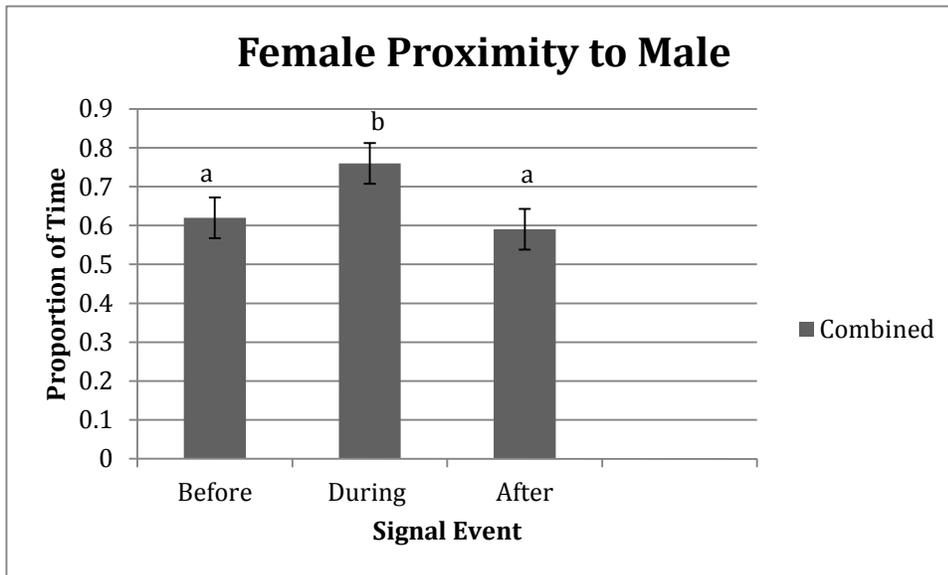
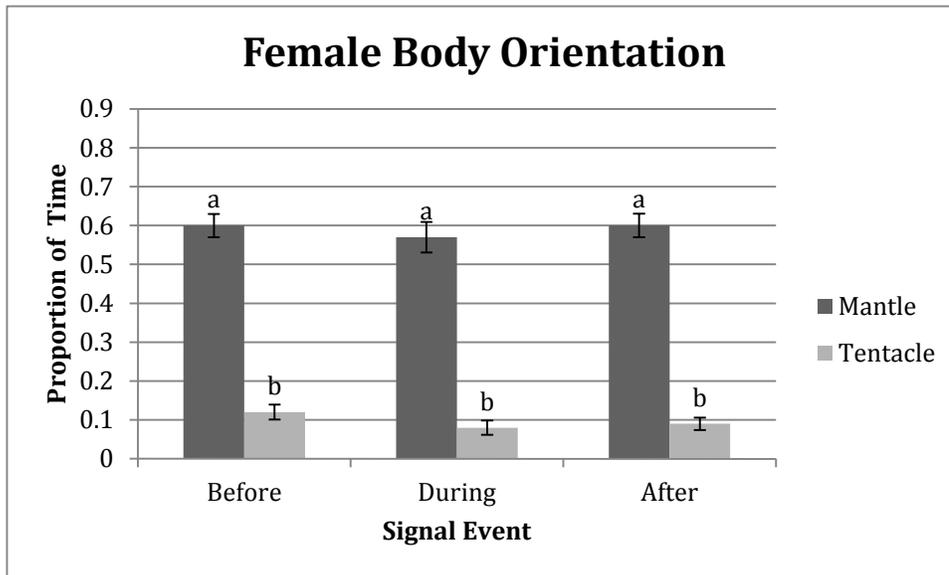


Figure 3c. Relationship between the average proportion of time males displayed DB, TMV, TMR and AMB and the number of contact events in mixed trials.



*Figure 4.* The mean proportion of time that females spend within one (female) body length of the male *before*, *during*, and *after* a male sexual signaling event, in female and mixed social contexts combined. Letters mark pair-wise significance, such that proportions labeled with the same letter are not significantly different. Standard error bars are shown around the mean.



*Figure 5.* The mean proportion of time females presented either their mantle or tentacles to the male *before*, *during*, and *after* male sexual signaling in female and mixed social contexts combined. Letters mark pair-wise significance, such that proportions labeled with the same letter are not significantly different. Standard error bars are shown around the mean.

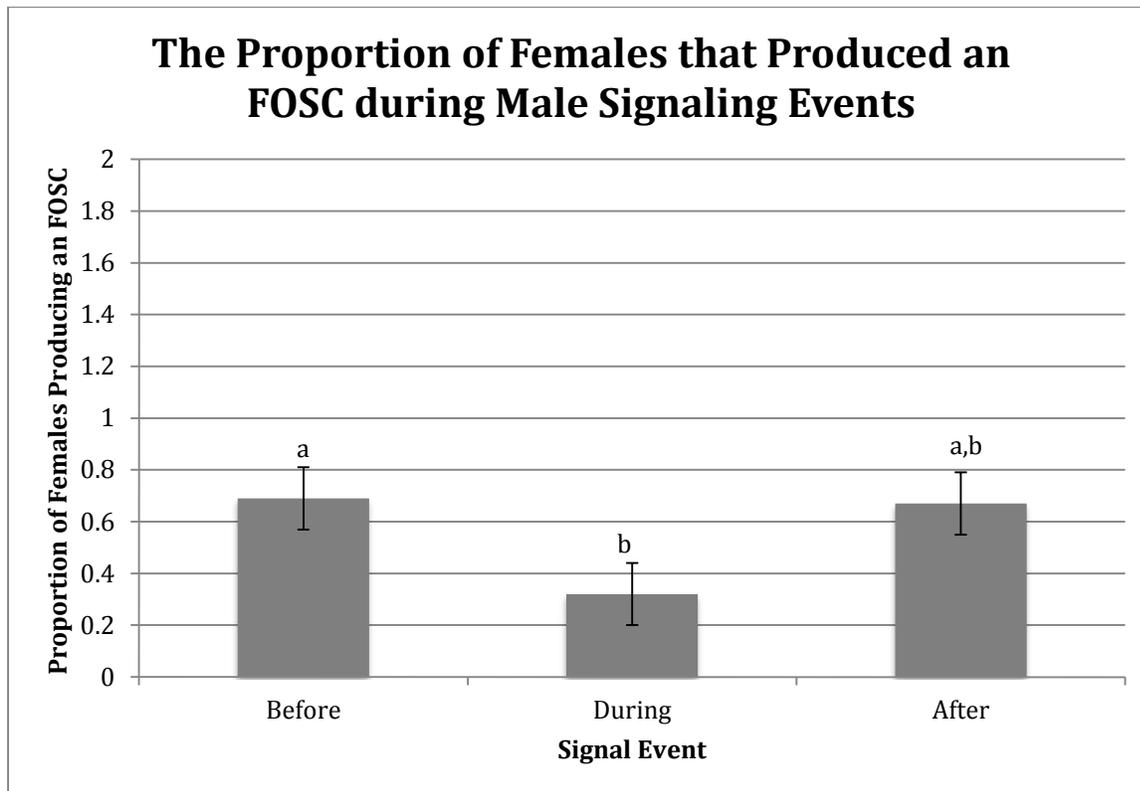


Figure 6. The proportion of females that produced FOSCs *before*, *during* and *after* male sexual signaling in female and mixed social contexts combined. Letters mark pair-wise significance, such that proportions labeled with the same letter are not significantly different. Standard error bars are shown around the mean.